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Progression

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<b>13. ABSTRACT (Maximum 200 Words)</b> Previous analyses suggested that loss of the transforming acidic coiled coil gene 2 (TACC2) occurs during breast tumorigenesis. TACC2 induced inhibition of the tumorigenic phenotype of certain breast cancer cells is mediated via the conserved TACC domain. This region binds to the histone acetyltransferases (HATs), hGCN5, and pCAF. We have now shown that hGCN5 is also downregulated in breast cancer, and that introduction of hGCN5 into cell lines can inhibit cellular division. Significantly, TACC2 can negate the <i>in vitro</i> suppression of DNA-dependent protein kinase (DNA-PK) mediated pCAF activity. This suggests that defects in TACC2 may impact chromatin structure, DNA repair and gene transcription. Thus, we have begun to address the potential interface between TACC2 and BRCA1 mediated regulation of the p21 gene. Our initial data suggests that basal levels of p21 and STAT1 are decreased in TACC2 transfected cell lines, resulting in defective p21 induction by interferon gamma. TACC2 does not directly bind BRCA1, but may compete with BRCA1 in a ternary complex with FHL2, a promoter specific coregulator. Combined, the data generated in this proposal indicates an intimate role of TACC2 at multiple levels in cell signaling pathways regulating mammary gland development and tumorigenesis.				
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## INTRODUCTION

The human transforming acidic coiled-coil (TACC) family of genes map to chromosomal regions associated with the development and progression of cancer (1,2). TACC2 is normally expressed at low levels in normal breast cells (3). Recently, Chen et al (3) demonstrated that TACC2 mRNA was downregulated in the more malignant clones of the HMT-3522 cell line based model for breast tumor progression, suggesting a role for TACC2 in malignant development. Little is currently known about the normal function of TACC proteins in the cell. However; there is significant evidence to indicate that TACC2, and TACC proteins, in general, are distributed both in the cytoplasm and the nucleus (4), with some of the nuclear localized TACC protein concentrated in nuclear speckles. This indicates that both the TACC proteins and histone acetyltransferases are physically located in the same subcellular compartment. This research project set out to determine whether TACC2 has breast tumor suppressor activity and possible mechanisms by which inactivation could promote tumor development.

This is the final report for this grant.

## BODY

**Specific Aim 1: Analysis of the effect of full length TACC2 and deletion mutants on growth suppression of breast cancer cell lines.**

### **Task 1: Analysis of the effect of full length TACC2 on growth of breast cancer cell lines**

To assess the potential growth suppressive role of the short TACC2 isoform (the major form expressed in the mammary gland) in breast cancer, we repeated the experiments of Chen et al in two different human breast cancer cell lines. To investigate the consequences of increased expression levels of TACC2, we introduced a plasmid construct (EGTACC2), which expresses the short TACC2 open reading frame fused to the green fluorescent protein (EGFP) into MDA-MB-468 and MCF7. The construct was transfected into each cell line, and stable cell lines selected as previously described. Increased expression of TACC2 alters the *in vitro* cellular dynamics of breast cancer cell lines in an apparently cell type specific manner. While TACC2 expression does not appear to inhibit cellular division, TACC2 does affect anchorage independent growth and cell migration. This implies that functional inactivation of TACC2 is important in the development of breast tumor metastases, which are the main cause of death in patients. These findings were published in Reference (5).

### **Task 2 Construction of deletion mutants of TACC2 and analyze their effect on breast cancer cell lines**

The finding by Chen et al that the carboxy terminal 571 amino acids of the TACC2 protein could partially reduce the malignant phenotype of one breast cancer cell line suggested that the carboxy terminal TACC domain could be a major determinant of the suppressive effects of TACC2. We have generated three N-terminal deletions and one carboxy-terminal deletion mutants in the mammalian expression vectors pcDNA3, EGFP-C2, and retroviral constructs. Unlike, the full length TACC2s construct, stable transfectants carrying the C-terminal deletion showed no significant alteration in their ability to form colonies in soft agar and were able to invade and migrate through the Matrigel matrix at levels similar to vector controls. For the N-terminal deletion constructs, no transfectants stably expressing the TACC2 truncation proteins were obtained. Using the pLPLNCX-TACC2 retroviruses, we infected 8 cell lines: MCF10A, MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-453, MDA-MB-468, Sk-Br3, T47D (and control mouse fibroblasts). Introduction of the coiled coil into any of the cell lines

was cell lethal, and we were unable to identify any clones expressing this domain. However, we were able to generate a small number of clones expressing the region from the SDP repeat motif to the end of the coiled coil (SDP-CC) in MCF10A and the murine fibroblasts, and this was higher than the number obtained from the full length TACC2. In no case could we obtain TACC2 expressing clones from the other cell lines. This indicates that the coiled coil domain is the major determinant of the TACC2 inhibitory effect, but that this may be ameliorated, in part by the inclusion of the SDP repeat domain.

**Specific Aim 2: Examination of the role of hGCN5 and the TACC2-hGCN5 interaction in breast tumorigenesis.**

**Task 3: Expression analysis, phosphorylation status and HAT activity of native hGCN5 in breast cancer cell lines**

To examine a potential role for hGCN5 in breast cancer, we performed Western blot analysis of 10 breast cancer cell lines, using a commercially available hGCN5 antibody. This showed that hGCN5 was expressed at relatively low levels in breast cancer cell lines. However, the closely related gene, pCAF is expressed in the same lines and we hypothesized that pCAF may also represent an alternative target for potential TACC2 mediated repression events. This finding negated further analysis of the phosphorylation status and HAT activity of native hGCN5. However, the low levels of expression raised the possibility that hGCN5 gene may be negatively regulated or even mutated in breast cancer.

**Task 4/5: Construction of hGCN5, and deletion mutants to determine their effect on breast cancer cell lines**

We have performed a detailed analysis of the role of hGCN5 in breast cancer cells. We cloned hGCN5 and a series of deletion constructs spanning the HAT domain, and/or the ADA2 interaction domain and the C-terminal bromodomain into the retroviral vector pLPLNCX. In each case, the hGCN5 construct was fused to green fluorescent protein, in order to readily confirm infection efficiency. We have used these pLPLNCX-hGCN5 retroviruses to infect the several breast cancer cell lines, including MCF10, MCF7 and MDA-MB-468, and selected for integration of the retroviral construct with neomycin. In all cases, excluding the retrovirus containing the N-terminal region of hGCN5 (DeltaHAT-ter), relatively few clones survived. Furthermore, we detected relatively little expression of the hGCN5 constructs in those stable clones produced, suggesting selection against hGCN5 expressing clones. In combination with the previous observation that lack of expression of hGCN5 in breast cancer cells, this data further indicates that hGCN5 may be a breast tumor suppressor gene in its own right.

**Task 6: Effect of hTACC2 expression on hGCN5 phosphorylation and HAT activity**

Task 6 initially included the effect of TACC2 on the phosphorylation status and HAT activity of native hGCN5. With the technical difficulties experienced in the analysis of hGCN5 in breast cancer cell lines, and with the commercial availability of newly developed resources to examine the pCAF protein *in vitro*, we have performed *in vitro* analysis of the effect of TACC2 on the histone acetyltransferase (HAT) activity of pCAF. To examine the potential significance of pCAF as an alternative target for TACC2 mediated repression events in breast cancer, we performed co-immunoprecipitation analysis using an antibody specific for native pCAF. Consistent with our prediction, native TACC2s (and TACC3) was found in the complex containing endogenous pCAF.

TACC2s binds to the HAT domain as well as the ADA2 interaction domain of hGCN5 and pCAF (6). As detailed in the second year report, *in vitro*, this interaction had no direct effect on the acetylation of histone H4 up to a 12 molar excess of TACC2s to pCAF, suggesting that, at least for this HAT member, TACC2s is neither a direct accessory component nor a competitive inhibitor which causes steric hindrance at the HAT catalytic site. Using an adaptation of the *in vitro* assay system previously used by Barlev et al (7), we found that activated DNA-PK reduces the histone acetylase activity of pCAF by approximately 25% ( $p < 0.05$ ). This indicated that, similar to hGCN5L2, DNA-PK negatively regulates pCAF HAT activity. It should be pointed out that these inhibition levels are less than that seen for hGCN5L2 (7). This may in part be accounted for by subtle differences in the tertiary structure of the proteins themselves, and/or the differences in assay systems used. Under these assay conditions, we found that introduction of exogenous TACC2s can negate this effect and completely restore the pCAF HAT activity. Thus, although TACC2 binding to pCAF may cause steric hindrance or conformational changes in pCAF, which prevent DNA-PK from phosphorylating the HAT domain, this does not prevent the physical interaction of pCAF with its histone substrate.

Intriguingly, an article was recently published that indicated that acetylation of Ku70 by histone acetyltransferases could be important in regulation of Ku70 subcellular localization and alter BAX mediated apoptosis (8). This suggests that TACC2s may act to alter the dynamic relationship between Ku70 and HATs in gene transcription and regulation of apoptosis.

Characterization of the interaction and colocalization of TACC2 with the histone acetyltransferases was published in Reference (6). Work on the regulation of the HATs will be submitted early next year.

### **Specific Aim 3. Characterization of the effect of TACC2 on the transcriptional enhancement of BRCA1 by CBP/p300.**

#### **Task 7: Detection of TACC2 and hGCN5 in the CBP/p300/BRCA1 complex**

We have demonstrated that TACC2 is found in a complex with CBP/p300 in the HEK293 cell line. Thus, we proposed that TACC2 and hGCN5 may be present in the complex of CBP/p300 and BRCA1. Downregulation of TACC2 or hGCN5 in breast cancer may therefore represent an alternative mechanism of inactivation of BRCA1 function, comparable with that seen in hereditary breast cancer. To avoid potential artifacts caused by overexpression of the target proteins, we have attempted to immunoprecipitate the native TACC2 and BRCA1 proteins in breast cancer cells. In addition we have performed analysis of the interaction between the proteins *in vitro* using GST pull down analysis. To date, this has failed to demonstrate a direct interaction between these proteins. However, recent data from large scale protein interaction studies in *C. elegans* has confirmed a direct functional interaction of the *C. elegans* TACC protein with the *C. elegans* homologue of the BRCA interacting protein BARD-1 (9). Furthermore, we have recently identified a functional interaction between TACC2 and FHL2 (four and half LIM domain protein 2), which is also known to interact with BRCA1 (10). We have been able to detect small amounts of TACC2 in the BRCA1 immunoprecipitate, suggesting that TACC2 indirectly interacts with BRCA1 via BARD1 or FHL2.

#### **Task 8/9: BRCA1 plasmid construction, determination of the role of TACC2 in the BRCA1 mediated regulation of the p21 promoter**

In our original proposal, we intended to examine the effect of TACC2 on GAL4-BRCA1 mediated induction of a GAL4 responsive expression cassette containing the chloramphenicol acetyl transferase



gene. As BRCA1 has now been shown to enhance the IFN- $\gamma$  mediated induction of the cyclin dependent kinase p21WAF1 (11), we have sought to investigate the direct effect of TACC2 on this BRCA1 regulated promoter. We determined that TACC2 reduces the basal level of this promoter in HEK293 cells. Furthermore, p21 levels are reduced in breast cancer cell lines stably expressing TACC2 from a CMV promoter (Fig 1). In an Affymetrix gene chip experiment (funded by the Roswell Park Alliance Foundation), we have independently determined that TACC2 downregulates the basal transcription level of the STAT1 $\alpha$  gene. As this protein is required for IFN- $\gamma$  mediated induction of p21, we performed a Western blot to determine whether p21 downregulation correlates with reduced STAT1 $\alpha$  protein. This does indeed occur (Fig 1) indicating that IFN- $\gamma$  signaling could be compromised in these cells. In MCF7 cells, we determined the effect of transiently upregulating TACC2 on the induction of p21 by IFN- $\gamma$ . In keep with the inhibition of STAT1, this resulted in inhibition of the IFN- $\gamma$  response. The significance of this result is currently unclear, but may be an artefact of overexpression, or alternatively, an as yet uncharacterized role of the FHL2 protein in the regulation of BRCA1 mediated events and/or regulation of STAT1. The possible interference of TACC2 with Ku70-mediated phosphorylation events in transcriptional control may also have multiple effects on other transcription factors controlling p21 transcription, as well as other uncharacterized promoters.

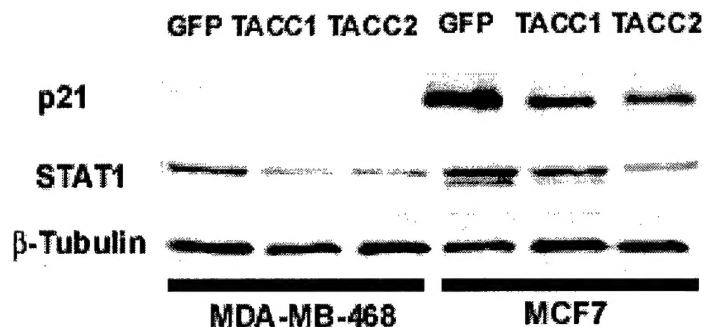


Fig 1. Western blot analysis of p21 and STAT1 protein expression in breast cancer cell lines transfected with TACC1 or TACC2 expression plasmids. This indicates that basal expression of p21 is reduced in TACC overexpressing cell lines. This appears in part to be due to a reduction in the basal level of STAT1.

## KEY RESEARCH ACCOMPLISHMENTS

- 1) Characterization of the tumor suppressor activity of TACC2
- 2) Demonstration that inactivation of hGCN5 occurs in breast cancer
- 3) Inhibition of proliferation of breast cancer cells by hGCN5 is mediated by the HAT and ADA domain.
- 4) Identification of TACC2s as a modulator of Ku70 induced inhibition of pCAF
- 5) Implication of TACC2 in BRCA1 mediated events by an indirect interaction with FHL2 and/or BARD1
- 6) TACC2 lowers the basal expression of p21, and the STAT1 $\alpha$  gene in MCF7 and MDA-MB-468
- 7) Overexpression of TACC2 inhibits p21 induction by interferon gamma.

## REPORTABLE OUTCOMES

- 1) Development of retroviral constructs expressing TACC2s, hGCN5 and specific subdomains
- 2) Development of *in vitro* methods to assess the effect of TACC2s on the Ku70-pCAF interaction

### Publications

- 1) Lauffart, B, Gangisetty, O, and Still, IH. (2003) Molecular cloning, genomic structure and interactions of the putative breast tumor suppressor TACC2. *Genomics* 81: 192-201
- 2) Gangisetty O, Lauffart B, Sondarva, G, Chelsea D, and Still IH. (2004). The Transforming acidic coiled coil proteins interact with nuclear histone acetyltransferases. *Oncogene* 23:14:2559-2563.

### Meeting Abstracts

- 1) Still IH, Lauffart B, and Gangisetty, O (2002). Analysis of the transforming acidic coiled coil 2 (TACC2) in breast cancer. (Department of Defense Era of Hope 2002)
- 2) Gangisetty, O., Sondarva, G., Vettaikkorumakankauv, A., Jaisani, Z., Lauffart, B., and Still, IH (2003). The transforming acidic coiled coil proteins are components of multiple protein complexes. (American Association for Cancer Research 94th Annual Meeting)

## CONCLUSIONS

In conclusion, we have analyzed the effect of the novel tumor suppressor gene, TACC2 and its histone acetyl transferase binding partners, hGCN5 and pCAF, on the cellular dynamics of breast cancer cell growth. We have determined that the expression of full length hGCN5 as well as constructs containing the histone acetyltransferase catalytic site are detrimental to breast cancer proliferation. In combination with the previous observation that breast cancer cells do not express significant levels of hGCN5, this data further indicates that hGCN5 may be a breast tumor suppressor gene in its own right. It appears that TACC proteins perform an assembly or coordination function bringing elements of the chromatin remodeling, transcriptional and posttranscriptional machinery together in the nucleus. In part this may also be due to the TACC2 protein in these complexes counteracting a negative modulator of histone acetylation activity. TACC2 can reduce the basal level of p21, in part through downregulation of STAT1. This would appear to counter our previous hypothesis that TACC2 is an activator of growth inhibitory pathways in the normal breast epithelium. However, the possible interference with Ku70 mediated phosphorylation events, and/or the interaction with FHL2 may have multiple effects on other transcription factors controlling p21 transcription, as well as other uncharacterized promoters, controlling mammary development and tumorigenesis.

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## PERSONNEL

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Melissa Cincotta, B.S.

**APPENDIX**

Lauffart,B., Gangisetty, O., and Still, I.H. (2003). Molecular cloning, genomic structure and interactions of the putative breast tumor suppressor TACC2. *Genomics* 81: 192-201

Gangisetty O, Lauffart B, Sondarva, G, Chelsea D, and Still IH. (2004). The transforming acidic coiled coil proteins interact with nuclear histone acetyltransferases. *Oncogene* 23:14:2559-2563

## Molecular cloning, genomic structure and interactions of the putative breast tumor suppressor TACC2

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### Abstract

The human transforming acidic coiled-coil 2 (*TACC2*) gene has been suggested recently to be a putative breast tumor suppressor. Now we can report the cloning of full length *TACC2* cDNAs corresponding to the major isoforms expressed during development. The *TACC2* gene is encoded by 23 exons, and spans 255 kb of chromosome 10q26. In breast cancer cell lines, *TACC2* is expressed as a 120 kDa protein corresponding to the major transcript expressed in the mammary gland. Although only slight differences in the expression of *TACC2* in normal versus breast tumors were observed, overexpression of *TACC2* can alter the in vitro cellular dynamics of some breast cancer cell lines. Significantly, we demonstrate that *TACC2* interacts with GAS41 and the SWI/SNF chromatin remodeling complex. This suggests that defects in *TACC2* expression may affect gene regulation, thus contributing to the pathogenesis of some tumors.

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**Keywords:** TACC; Breast cancer; Chromosome 10; GAS41; INI-1; SWI/SNF

### Introduction

The identification of potential oncogenes and tumor suppressor genes is important in the elucidation of the pathways that govern cell growth, division, and differentiation. Recently, we identified a family of evolutionary conserved genes, the *TACC* (transforming acidic coiled coil)-containing family, which are expressed in a temporal and tissue-specific pattern during development [1,2]. The proteins encoded by these genes are highly acidic and contain a conserved 200 amino acid coiled-coil domain, the *TACC* domain, which is predicted to play an important role in the function of these proteins [1,2].

Growing evidence supports a role for the *TACC* family in processes underlying the development of cancer. The human *TACC* orthologues map to regions associated with tumorigenesis and progression. *TACC1* and *TACC2* are located in chromosome 8p11 and 10q26, respectively, two regions that are implicated in breast and other tumors [1,2], while *TACC3* maps to 4p16, within 200kb of a translocation

breakpoint cluster region associated with multiple myeloma [2]. In vitro and in vivo studies also indicate that the *TACC* proteins are linked to the processes of cell growth and differentiation. *TACC1* and *TACC3* are expressed at high levels during embryogenesis and are then downregulated in differentiated tissues, being expressed either at low levels, or only in restricted tissues [1–3], suggesting that they may play a role in the processes that promote cell division before the formation of differentiated tissues. The essential role of one of the *TACC* genes, *TACC3*, in development has recently been demonstrated, because homozygous knockout mice die during mid to late gestation [4].

Recently, in the HMT-3522 cell line-based model for breast tumor progression, Chen and colleagues [5] demonstrated that *TACC2* mRNA is downregulated in the more malignant clones of the series. These authors also reported the cloning of a 3.8 kb *TACC2* cDNA (named AZU-1), encoding a protein of 571 amino acids and predicted molecular mass 64 kDa of these malignant cells. Reintroduction of this cDNA into the malignant breast tumor cells reduced their ability to grow and metastasize [5]. Therefore, this suggested that *TACC2* is a breast tumor suppressor gene, the downregulation of which is an important step

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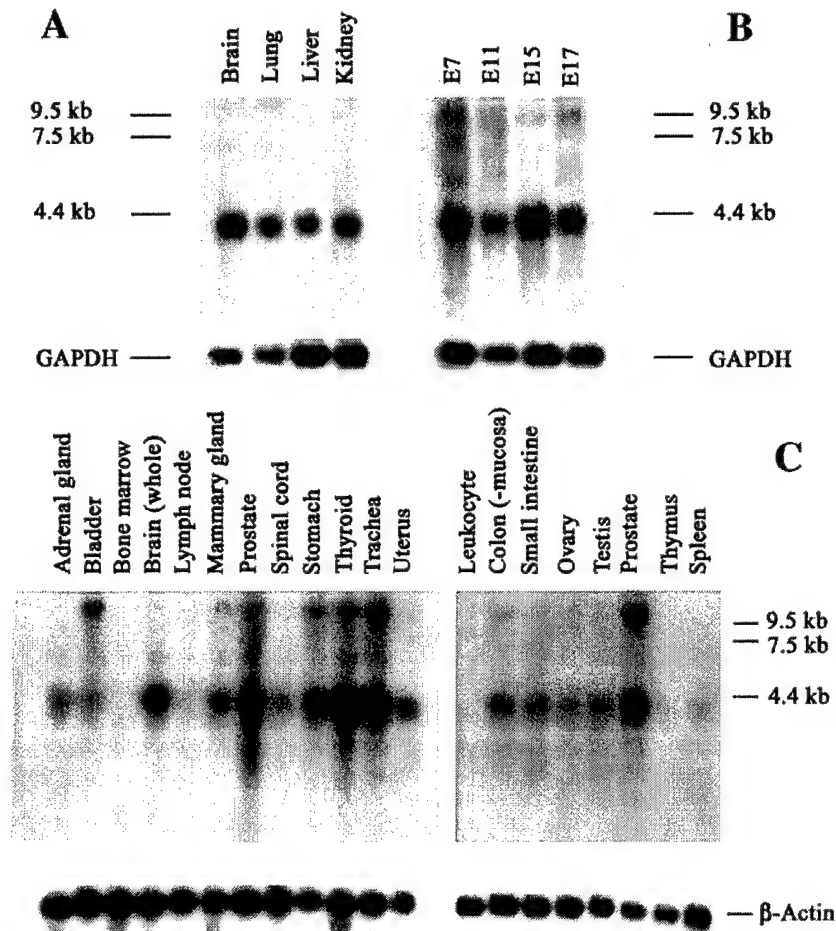


Fig. 1. Northern blot analysis of TACC2. (A) The predominant TACC2 transcript expressed in human fetal tissues is 4.2 kb long. (B) During murine embryogenesis, the 4.2 kb transcript is the major isoform. A minor 9.7 kb transcript is also detected. (C) In adult tissues, the most common transcript detected is the 4.2 kb transcript. The 9.7 kb transcript is detected at relatively high levels in trachea. TACC2 is not significantly expressed in tissues of immune and hematological origin.

during breast tumorigenesis. *TACC2* was also recently identified as an erythropoietin-inducible gene in human microvascular endothelial cells [6]. In this case, a 3.3 kb cDNA was described (named ECTACC) that showed significant nucleotide identity with the AZU-1 sequence, but that encoded a larger protein with a predicted molecular mass of 100 kDa.

In this report, we describe the cloning and expression of the *TACC2* gene. We have characterized the major isoforms of TACC2 proteins expressed during development, and identified the splice variant expressed in the mammary gland. We demonstrate using the available genomic and cDNA sequence data that both AZU-1 and ECTACC may contain cloning artifacts, thereby explaining the discrepancies in their sequences. In light of these findings, we have reevaluated the potential role of *TACC2* as a breast tumor suppressor gene. We also show that, similar to TACC1, the TACC2 protein binds to GAS41, and forms a complex with the SWI/SNF core subunit INI-1. This suggests that the

suppressive effects of TACC2 may be mediated by interactions with a potential transcription factor complex.

## Results

### Northern blot analysis of TACC2 in human and mouse tissues

During the cloning of TACC1, we isolated a 1 kb clone, FL4, that was highly related to the 3' end of the TACC1 open reading frame [1], and thus defined a new gene, which we termed *TACC2* [1]. As a first step toward the characterization of this member of the *TACC* gene family, we performed Northern blot analysis of human and murine tissues using a TACC2-specific probe (Fig. 1). A single 4.2 kb transcript was detected in the human fetal tissues tested (Fig. 1A). This transcript was also detected at all stages of murine embryonic development, in addition to a lower

abundance transcript of approximately 10 kb (Fig. 1B). Both transcripts were evident in a number of adult human tissues, although the relative abundance of each varied depending on the tissue. For example, the 4.2 kb transcript was the predominant isoform in most TACC2-expressing tissues, and is highly expressed in whole brain, as well as prostate, thyroid, and trachea. The larger transcript is expressed at lower levels in a number of tissues, but is a major RNA species in the bladder (Fig. 1C), placenta, skeletal, as well as heart muscle (data not shown). TACC2 does not appear to be expressed at significant levels in the bone marrow, lymph node, or in peripheral blood leukocytes.

#### *Cloning of the transcripts corresponding to the major forms of TACC2*

A BLAST search with the FL4 sequence originally identified a single UNIGENE cluster of cDNAs, HS90415, which included approximately 2.4 kb of the 3' end of the TACC2 transcript. To determine the full sequence of the 4.2 kb major transcript, we used FL4 to screen a fetal brain library to isolate additional TACC2 cDNAs. Sequence analysis of these clones extended the TACC2 sequence and identified an additional human UNIGENE cluster HS202303. A 3,686 bp contig for the human fetal cDNA (GB:AF095791) was assembled, but it could not be extended farther 5', either through cDNA library screening or 5'RACE. During this analysis, two laboratories independently reported the cloning of cDNAs corresponding to TACC2. The GB:AF220152, named ECTACC, is 3311 bp long with a 5' untranslated region (UTR) of 369 bp encoding a protein of 906 amino acids [6]. A second TACC2 cDNA, named AZU-1, contained an open reading frame (ORF) of 571 amino acids, with a 5' UTR of approximately 1.6 kb [5]. However, the ORF of this latter cDNA could be extended 1.3 kb upstream of the proposed start methionine of AZU-1, in agreement with the predicted protein sequences from ECTACC and AF095791. This suggested the possibility that the AZU-1 cDNA represents a RACE cloning artifact, or corresponds to an incomplete splicing product from the other TACC2 isoforms.

We have previously shown that the *TACC2* gene is located on chromosome 10q26, in the same interval as *FGFR2* [1]. Examination of the public human genome database revealed that the available TACC2 sequence was contained within a single BAC clone, RP11-296H2 (GB:AL135793). A comparison of the sequence of this BAC with AF095791 and AF220152 revealed that the two cDNAs diverge upstream of a potential splice acceptor (Fig. 2). Similarly, the AZU-1 nucleotide sequence diverged upstream of another potential splice acceptor. Sequences identical to the 5' region of AZU-1 are also present in two cDNAs in the UNIGENE database, and BAC RP11-296H2, suggesting that they may correspond to additional exons of TACC2. However, because these putative upstream exons

contained stop codons in all three reading frames, and we failed to identify them in rt-PCR analysis from breast, colon, or prostate RNA, it appeared likely that they did not represent the legitimate 5' end of the TACC2 cDNA.

Analysis of the genomic sequence within 200 kb of the 5' region of the cloned TACC2 sequence revealed two hypothetical genes, Hs10\_27257\_29\_3\_1 and Hs10\_27257\_29\_3\_2 with predicted transcript sizes of 5673 nt and 1491 nt, respectively (Fig. 2). When these predicted transcripts were used to search the EST database, several cDNAs were identified that not only linked the two predictions, but also, through cDNA sequences BG822027, BF992557, and BF36966, linked Hs10\_27257\_29\_3\_2 to the TACC2 UNIGENE cluster HS272023, which suggested that the TACC2 transcription initiation start is located approximately 150 kb upstream of the 5' sequence of AF095791 and AZU-1. To confirm that the predictions did correspond to a bona fide TACC2 transcript, we next designed a series of primers for use in rt-PCR. Overlapping PCR products stretching the length of the genescan/TACC2 predictions were amplified from colon, prostate, and mammary gland RNA. Sequence identity of these products was confirmed and the complete sequence generated by this analysis corresponded to a transcript of 9773 bp, which is similar to the length of the largest TACC2 transcript (TACC21) identified by Northern blot analysis. Using primer combinations T2F77/T2R6558 and T2F340/155R, rt-PCR analysis of mammary gland RNA revealed that the smaller 4.2 kb transcript (TACC2s) was produced by the alternative splicing of this product, mainly resulting from the exclusion of a 5313 bp exon (see below). Thus, the main form of TACC2 expressed in the mammary gland is 4146 bp long. The sequences of these two forms have been deposited in GenBank with the accession numbers: AF528098 (TACC21 isoform) and AF528099 (TACC2s isoform).

#### *TACC2 protein structure*

The two major transcripts of human *TACC2* are 4.2 kb long and approximately 9.7 kb long. They encode predicted proteins of 1094 amino acids and 2948 amino acids with molecular masses of 119 kDa and 310 kDa, respectively. The size of both TACC2 proteins is significantly larger than that of TACC1 (805 amino acids) and of TACC3 (838 amino acids) [1,2]. Similar to the two other human TACC family members, both TACC2 proteins are highly acidic with a pI of 4.6 and 4.9, respectively, being enriched in acidic (16.2% and 15.3%), proline (10.4% and 10.4%), and serine (11% and 11.2%) residues. Both products contain a number of strong PEST sequences, suggesting that the TACC2 protein(s) may be subject to regulated degradation. This may explain the ladder of multiple smaller forms of the TACC2 protein ranging in size from 120 kDa–60 kDa, originally detected by other groups using Western blot analysis [5,7]. TACC2 also contains several basic regions, which may serve as nuclear localization signals. Indeed, we

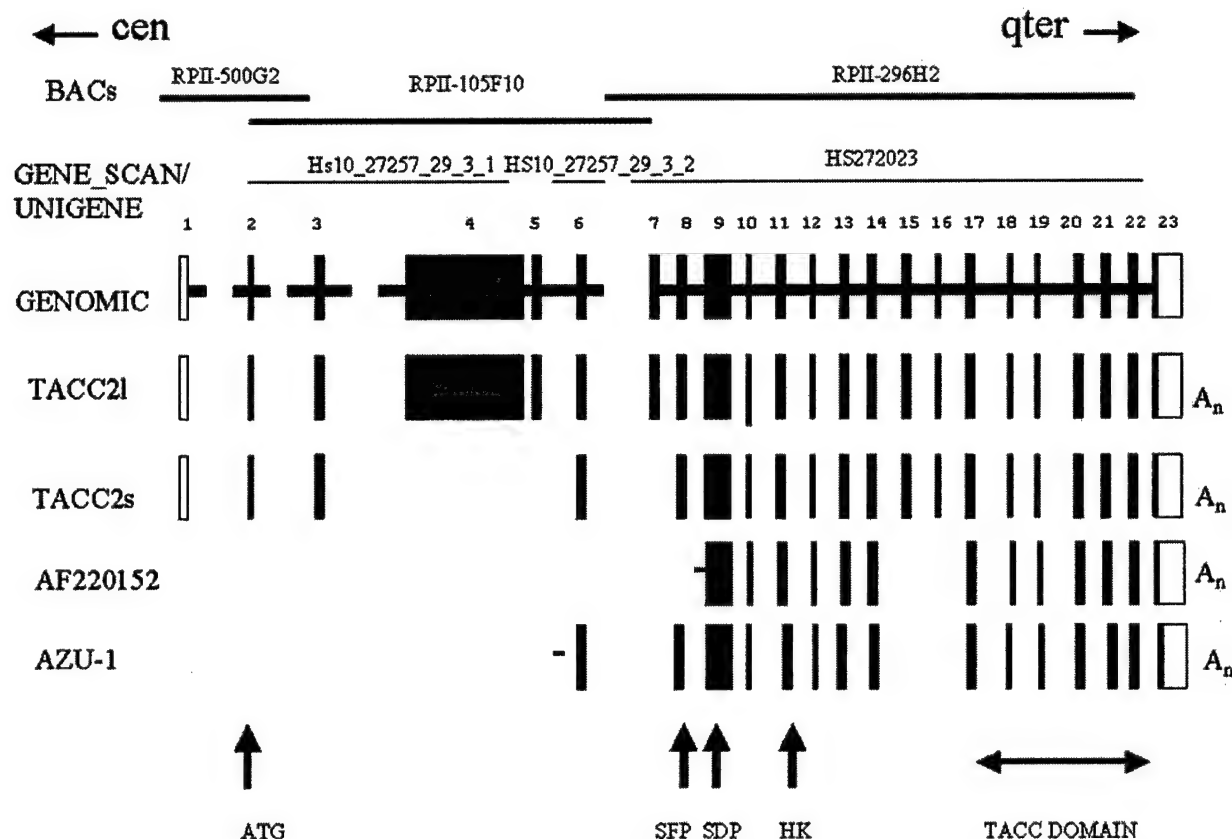


Fig. 2. Genomic structure of *TACC2*: Comparison of the previously cloned *TACC2* cDNAs, genome scan predictions, the *TACC2* cDNAs reported in this manuscript, and the genomic structure of *TACC2*. Exons are depicted as black boxes with introns as intervening lines. The SFP motif is located in exon 8. PEST sequences are spread over exon 8 and 9, the two SDP repeats are located in exon 9, and the HATDEEKLA (HK) motif in exon 11. The coiled-coil domain of *TACC2* is dispersed over exons 17–23.

have shown that, similar to all the TACC proteins, *TACC2* can also be detected in the nucleus, as well as the cytoplasm of interphase cells [7]. Thus, localization of *TACC2* to the nucleus may be regulated by the use of these signals.

As previously noted, the *TACC2* coiled-coil domain is highly related to the coiled-coil domains of *TACC1* (74% identity) [1], and to a lesser extent to *TACC3* (53% identity) [2], *Xenopus maskin* protein (56% identity) [8], and the D-TACC protein (26% identity, 55% similarity) [9]. Apart from the coiled-coil domain, the human TACC proteins share limited regions of homology. Both *TACC1* and *TACC2* contain a 16 amino acid SFP motif SSDSE-X<sub>2</sub>-FETPE-X<sub>2</sub>-TP, and an absolutely conserved string of nine amino acids, HATDEEKLA (Fig. 2). Neither of these motifs is found in other TACC proteins, or proteins unrelated to the TACC family, suggesting that they are likely to play a role specific to *TACC1* and 2. Additionally, *TACC2* contains two copies of the 33 amino acid SDP repeat, which are conserved between the members of the vertebrate TACC family [10]. We have recently demonstrated that the region of *TACC1* containing these repeats serves as the binding site for the SWI/SNF component/accessory factor, GAS41 [10].

#### Genomic organization and multiple splicing of the *TACC2* gene

Reconstruction of the *TACC2* transcript sequences and comparison with the genomic sequence of chromosome 10q26 revealed that the *TACC2* gene is spread over 23 exons, and is approximately 255 kb long (Table 1). The exons range in size from 61 bp (exon 10) to 5313 bp (exon 4), with introns ranging in size from 247 bp (intron 20) to more than 51 kb (intron 7). The intron/exon boundaries conform to the GT-AG splicing paradigm. The first exon comprises the majority of the 5' UTR, with the start methionine located in the second exon. The motifs, which are conserved in different TACC proteins, are also located in separate exons: the SFP motif in exon 8, the two SDP repeats in exon 9, and the HATDEEKLA motif in exon 11. The coiled-coil domain of *TACC2* is dispersed over the final seven exons, exons 17–23 (Fig. 2).

The major isoforms of *TACC2*, the 9.7 kb and 4.2 kb transcripts, mainly differ by the inclusion of the unusually large 5.3 kb exon 4. Analysis of the publicly available mouse genome database (contig NW\_000333) suggests that an analogous exon is present in the *TACC2* gene on mouse



Table 1

Details of the genomic structure of TACC2. The exon/intron structure, sequence of the intron/exon junctions, and the approximate size of the exons and introns are given

Exon	Exon size (bp)	3' Acceptor site	5' Donor site	Intron size (bp)
1	296	N/A	GTGCGgtaa	32446
2	79	ttcttcagTCACC	ACCAGgtgg	28418
3	113	cctggctcagAGGAC	GTCCAGgtag	32091
4	5313	atattttcagCATTG	TGACAGgtac	517
5	114	attatccagAGAGA	AGAAAGgtca	43659
6	126	ctctccccagTTCAC	CCCAGAGgtac	10871
7	135	gtgtccccagCATCT	CAGCAGgtat	51303
8	137	ctctcatcagGAGTT	AACCAGgtaa	15220
9	1312	ttgtttccagGATGT	AAGGACgtaa	3683
10	61	ttgtttccagTGACA	TCCCAGgtac	1176
11	202	ctctttgcagGACCC	CTGAGGgtaa	7898
12	62	tggtttgcagAGTTG	TTACCTgtaa	1579
13	116	gaccctgcagCAGGA	TTCAGGgtat	1356
14	172	cttccccagGTCAA	GAAATTgtaa	1338
15	141	cggctttcagACAGC	CTCCAGgttt	865
16	90	ctgtggcagGAGGA	GCCAAGgtacc	6955
17	144	gattccacagAGAGA	GCAGAGgtatt	423
18	77	ctgaaactagATCAT	AATGAGgtca	3921
19	44	tctcttatagGAAAA	TGATAGgtag	6642
20	161	ctcctgccagAGGAC	CGCAAGgtag	247
21	107	gctgtgtcagAATGA	GGACAGgtaa	388
22	121	gtctccgcagGGCCA	CAGAAAgtaa	4326
23	63bp+3' UTR	tcacttcagAATAA	—	—

chromosome 7 (data not shown). Additional alternative splicing is evident in the region preceding the coiled-coil motif. Both AF220152 and AZU-1, as well as associated cDNAs, are missing exons 15 and 16 (Fig. 2). We have performed rt-PCR analysis of this region, detected these shorter transcripts, and a transcript missing only exon 16 in a number of fetal and adult human RNA samples (data not shown). However, the significance of this alternative splicing is unclear, because these exons do not encode a known functional motif.

#### Expression of the TACC2 protein in breast cancer cells

Commercially available antibodies raised against TACC2-specific peptides have recently become available. The technical specification of antibody 07-228 (Upstate Biotech.) suggests that this TACC2 antibody recognizes a molecular species of 63 kDa in HeLa cells. However, in HEK293 cells (human embryonic kidney), this antibody detects a protein of 120 kDa (Fig. 3), which is in agreement with the predicted protein corresponding to the 4.2 kb transcript expressed in human fetal kidney (Fig. 1A). This isoform was also detected in protein extracts from normal brain, as well as a number of different cell lines (data not shown).

It has been suggested that TACC2 is significantly down-regulated in breast cancer cell lines relative to the immortal but not transformed breast epithelial cell line MCF10A [5]. With the observation that the TACC2 protein detected previously [5] may be the result of degradation or aberrant

splicing events, we analyzed by Western blotting the total protein extracts from 70% confluent cultures of breast cancer cell lines. TACC2 is predominantly expressed as the 120 kDa isoform in all breast cancer cell lines tested, although no dramatic difference in TACC2 protein levels could be detected at this level of resolution (Fig. 3A). TACC2 mRNA levels are also only slightly reduced in matched breast tumor compared to normal breast tissue pairs when a TACC2-specific probe was hybridized to a commercially available multiple cancer profiling array (BD Biosciences, Clontech, USA) (Fig. 3B), suggesting that only subtle, if any, changes in TACC2 expression may be present in breast tumors.

#### Assessment of TACC2 as a breast cancer growth suppressor

Previous analysis of the effect of TACC2 on the growth characteristics of breast cancer cells utilized the partial AZU-1 cDNA clone [5]. To determine whether the effects noted previously were due to the use of a partial clone, and therefore may be due to a dominant negative effect, we repeated the in vitro growth experiments of Chen and colleagues [5] in two different human breast cancer cell lines. To investigate the consequences of increased expression levels of TACC2, we introduced a plasmid construct (EGTACC2), which expresses the TACC2s isoform fused to the green fluorescent protein (EGFP) into MCF7 and MDA-MB-468, and stable cell lines were selected, as previously described [1]. The expression of the fusion protein

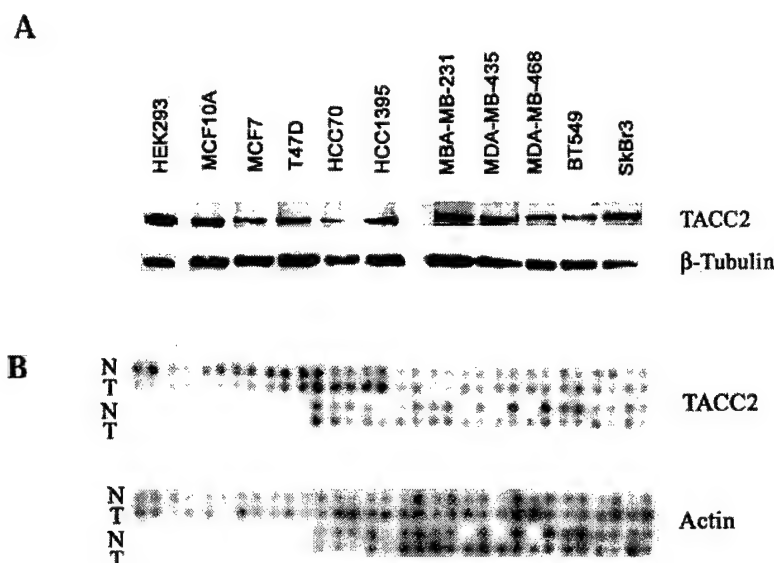


Fig. 3. (A) Western blot analysis of TACC2 protein expression in breast cancer cell lines. TACC2 is expressed as the 120 kDa isoform, corresponding to the predicted open reading frame from the 4.2 kb transcript. 10  $\mu$ g of protein derived from 70% confluent cultures was loaded in each lane of the Western blot analysis. Even loading was confirmed using a  $\beta$ -tubulin antibody. (B) Analysis of the expression of TACC2 mRNA in breast cancer samples using the Clontech Cancer Profiling Array. This blot contains SMART-amplified cDNA from tumor (T) and matched normal (N) tissues. The blot was also hybridized with actin to confirm even sample loading.

was verified by fluorescence (Fig. 4A), and Western blot analysis demonstrated correct and equivalent expression of the EGFP fusion protein in all cell lines (data not shown). In the cell proliferation assay, TACC2 overexpression reduced the growth rate of the transfected MDA-MB-468, but only slightly inhibited the growth of MCF7 within the assay period (Fig. 4B).

An important indicator of whether a gene product is a potential oncogene or tumor suppressor is its ability to impart, or abolish anchorage independent growth in vitro. Therefore, we determined whether transfection of EGTACC2 into MCF7 and MDA-MB-468 cells would alter cellular motility and growth in soft agar. TACC2 overexpression in three stable transfectants produced no significant alteration in the ability of MCF7 to form colonies in soft agar ( $P = 0.11$ ); however, in MDA-MB-468 the number of TACC2-overexpressing colonies was significantly reduced ( $P = 0.01$ ) when compared to controls (Fig. 4C). MCF7 has previously been shown to migrate poorly through a basement membrane matrix (Matrigel), and transfection of EGTACC2 into MCF7 failed to alter migration rates. However, EGTACC2/MDA-MB-468 transfectants were significantly impaired in their ability to invade and migrate through the Matrigel matrix ( $P = 0.001$ ) within a 24 hour period.

#### *TACC2 complexes with GAS41 and the SWI/SNF core component INI-1*

Both large and short isoforms of TACC2 contain two copies of the SDP repeat motif [10]. We have shown recently that the region of TACC1 containing these repeats

serves as the binding site for the SWI/SNF component/accessory factor, GAS41 [10]. The degree of conservation of this region between the TACC family members suggests that GAS41 may be a common binding factor for the TACC family [10]. To determine whether TACC2 could also bind this protein, we performed co-immunoprecipitation assays in the EGGAS41/HEK293 cell line that was used for the initial characterization of the GAS41-TACC1 interaction [10]. As shown in Fig. 5, GAS41 is found in a complex with TACC2s in these cells. To confirm further that the interaction between GAS41 and TACC2s is direct, we also performed in vitro GST-pull down assays. Fig. 5B clearly demonstrates that the interaction between GAS41 and TACC2s is direct, and can occur independently of any interaction with TACC1. Furthermore, immunoprecipitates of the SWI/SNF core subunit INI-1 in two breast cancer cell lines, MDA-MB-231 and MCF7, also contain TACC2, thus showing that TACC2 can, at least indirectly, associate with an INI-1 containing SWI/SNF complex (Fig. 5C).

#### Discussion

We have now reported the cloning, expression, and genomic organization of the *TACC2* gene. TACC2 is expressed as two major isoforms during development; the 4.2 kb transcript is the more abundant form, however, in differentiated tissues the relative levels of the two isoforms can be different, depending on the nature of the tissue. Both isoforms can be expressed in the same tissue, although each transcript can be expressed independent of the other. Thus,

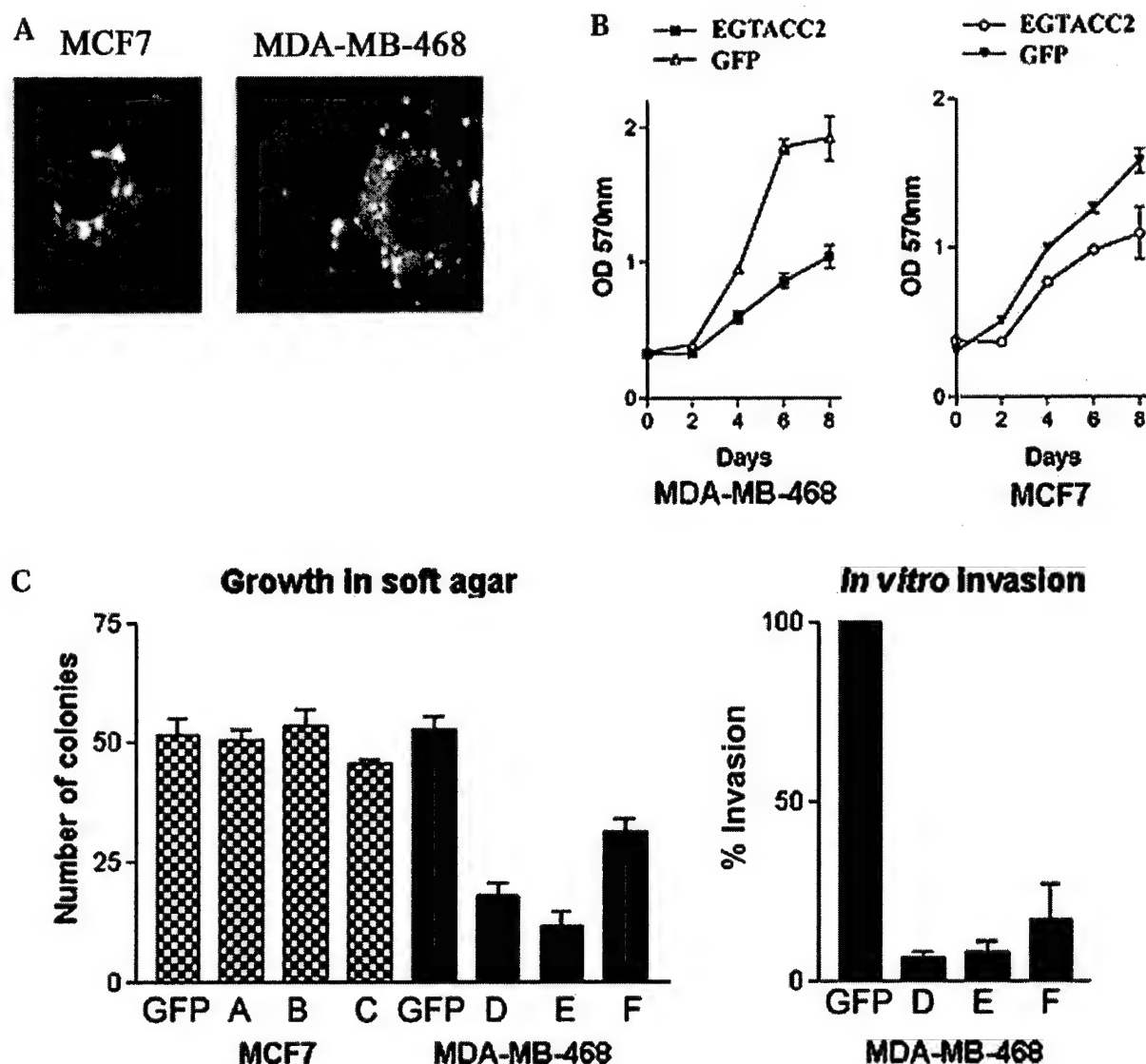


Fig. 4. Analysis of the effect of EGTACC2 expression in breast cancer cell lines. (A) Expression of EGTACC2 in representative live breast cancer cell lines as determined by fluorescence microscopy. Overexpression results in an accumulation of the fusion protein in the cytoplasm, as previously noted in other cell lines [7]. EGFP by comparison was uniformly distributed throughout the cell (data not shown). (B) Cell proliferation curves for EGTACC2 transfected MDA-MB-468 and MCF7. One stable transfectant line of each is shown. (C) The in vitro growth characteristics of TACC2 overexpressing MDA-MB-468 and MCF7 transfectant cell lines were analyzed as described in [5]. No significant difference between the formation of colonies in soft agar was noted for MCF7-EGTACC2 transfectants A–C ( $P > 0.05$ ) compared to the control MCF7 cells expressing EGFP alone. However, both the ability of MDA-MB-468 clones D–F to form colonies in soft agar and migrate through Matrigel was reduced by overexpression of TACC2 ( $P < 0.05$  in each case). Data points correspond to the mean of three experiments  $\pm$  SEM.

the 4.2 kb transcript is the predominant transcript in the mammary gland, but expression of the larger 9.7 kb transcript is increased in tissues that contain a high proportion of either smooth or striated muscle. Similar to the other TACC proteins, both transcripts encode highly acidic proteins, which are rich in acidic, proline, and serine residues. As previously noted [1,5], the TACC2 coiled-coil domain is most similar to TACC1 and, from database analysis, TACC2 has orthologues in mouse, *Xenopus*, and the pufferfish (Still and Liang, unpublished), suggesting that these genes are the result of a duplication event that occurred after

the invertebrate/vertebrate division, 600 million years ago. Apart from the coiled-coil domain, both TACC2 isoforms show isolated regions of homology with the other TACC proteins. Significantly, TACC2 contains two copies of the SDP repeat, which we recently identified as a binding site for the GAS41 protein [10]. Co-immunoprecipitation and in vitro analysis confirm that TACC2 also binds directly to this protein. GAS41 was originally believed to be a transcription factor [11,12], and, more recently, has been shown to interact directly with the INI-1 core component of the SWI/SNF chromatin remodeling complex [13]. We have now demon-

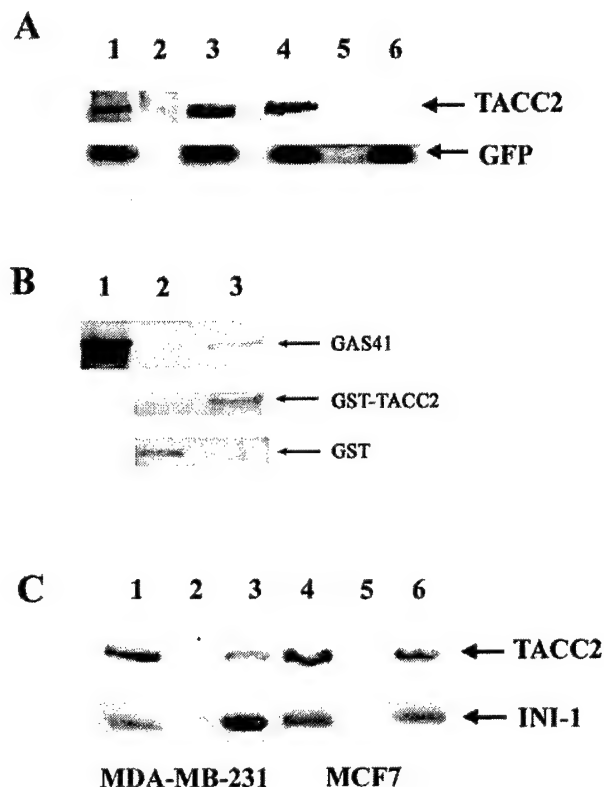


Fig. 5. Interaction between TACC2 and the SWI/SNF complex. (A) In vivo interaction of GAS41 with native TACC2s. Stable HEK293 transfectants expressing either GFP fused to GAS41 (EGGASB1) or GFP alone (EGFP/HEK293) were immunoprecipitated with either anti-GFP or rabbit IgG and immunoblotted with anti-TACC2 antibody. EGGASB1 and EGFP/HEK293 cells express the 120 kDa TACC2s isoform (lane 1 and 4) and this protein is specifically immunoprecipitated from EGGASB1 cells by the anti-GFP antibody (lane 3), but not control IgG (lane 2). No interaction between GFP and TACC2 was detected in immunoprecipitates of EGFP/HEK293 expressing GFP alone (lane 6). Lane 5 represents EGFP/HEK293 immunoprecipitated with control IgG. Bottom panels confirm that similar amounts of GFP-GAS41 (53 kDa) and GFP (27 kDa) are immunoprecipitated by the anti-GFP antibody in the lanes 3 and 6. (B) In vitro interaction of TACC2s and GAS41. Lane 1, 10% input of in vitro translated GAS41 protein; lane 2, GST as negative control; and Lane 3, GST-TACC2. Top panel: Autoradiograph of 8% SDS polyacrylamide gel with in vitro translated GAS41 protein pulled down with GST-TACC2. Bottom two panels represent Coomassie blue-stained gel of pull-down experiment demonstrating loading of GST fusion proteins. (C) Immunoprecipitation of native TACC2 by native INI-1 from breast cancer cell lines. TACC2 is expressed as the 120 kDa short isoform in MDA-MB-231 (Lane 1) and MCF7 (Lane 4). TACC2s is specifically immunoprecipitated by the anti-BAF47 (INI-1) antibody from both cell lines (Lanes 3 and 6), but not IgG (Lanes 2 and 5).

strated that TACC2 not only binds to the GAS41 protein, but can also be found in a complex with the SWI/SNF core subunit INI-1. A role for TACC proteins in transcriptional and post-transcriptional events has been shown for murine Tacc3 [3], and the *Xenopus* maskin protein [8], as well as implied for TACC1 [10,14]. This combined data suggests further that TACC proteins perform a scaffolding, assembly, or coordination function in bringing elements of the

chromatin remodeling, transcriptional, and post-transcriptional machinery together in the nucleus.

We have previously mapped TACC2 to chromosome 10q26, close to *FGFR2* [1]. The entire TACC2 gene spans 23 exons dispersed over approximately 255 kb. Surprisingly, the long and short TACC2 isoforms differ by the inclusion of an unusually large 5 kb exon in the larger transcript. Analysis of the publicly available mouse genome database suggests that a similar-sized exon is present in the TACC2 gene on mouse chromosome 7 (Still and Liang unpublished manuscript). The inclusion of large exons in TACC gene sequences appears to have occurred during the evolution of the TACC gene family. This is particularly evident when the human and mouse TACC3 sequences are examined [2]. In this case, a 996 bp exon has been acquired after the divergence of the primate and rodent lineages [2]. The significance of these sequences remains to be elucidated; however, large exons, such as exon 11 of *BRCAl*, which includes about 61% of the coding sequence of the gene, do appear to be hotspots for inactivating mutations in cancer [15]. TACC2 maps to a region of the genome that exhibits loss of heterozygosity and deletions in many tumors, including breast [16], glioblastoma [17–19], lung [20], respiratory tract [21], prostate [22], and endometrial cancer [23,24]. Clearly, an examination of this exon in tumors derived from tissue that express the long isoform of TACC2 might reveal that TACC2 mutations underlie some of these tumor types.

It has been proposed that TACC2 is a class II tumor suppressor in that changes in expression, as opposed to mutations, can be linked to breast tumor progression [5]. Although we have not detected major changes in TACC2 protein levels in breast cancer cell lines, or RNA levels in resected tumor samples, analysis of TACC2-overexpressing cell lines revealed effects on the ability of breast cancer cells to exhibit anchorage independent growth and to migrate through a basement membrane-like matrix. TACC2s had little effect on the growth characteristics of the estrogen receptor positive MCF7 cell line, but significantly reduced the ability of estrogen receptor negative cell line MDA-MB-468 to grow in soft agar and to migrate through an extracellular matrix. Interestingly, the HMT-3522 cell line model, in which a potential role of TACC2 in breast tumorigenesis was first examined [5], also lack estrogen receptors [25], which could suggest that the effect of overexpression of TACC2s on the ability of breast cancer cells to divide in culture may be differentially affected by the genetic background of the original tumor.

In summary, we report the cloning and expression of the second member of the TACC gene family. The major 4.2 kb TACC2 transcript, which is expressed in the mammary gland, encodes a protein of 120 kDa. We failed to detect significant differential expression of this TACC2 isoform in cell lines and resected tumors, compared to normal controls. Therefore, additional immunohistochemical analysis of surgically resected breast tumors will need to be conducted to

define more accurately a link between TACC2 and breast tumorigenesis. We have also demonstrated that TACC2 interacts with GAS41 and INI-1, which suggests that TACC2 could play a role in transcription through interaction with the SWI/SNF complex. A proposed role of TACC2 in transcriptional events may explain how a decrease in TACC2 expression could be associated with the progression to a more malignant phenotype in the HMT-3522 cell line model [5].

## Materials and methods

### *Expression analysis and cloning of the TACC2 cDNA*

The TACC2 cDNA clone, FL4, was originally isolated from a fetal lung cDNA library during isolation of the TACC1 cDNA [1]. A subclone of this cDNA, lacking the conserved coiled-coil sequence, was hybridized to commercial multiple tissue Northern blots (BD Biosciences Clontech, Palo Alto, CA, USA), according to the manufacturer's instructions. Additional cDNAs were identified by screening a human fetal brain cDNA library (BD Biosciences Clontech), according to standard protocols. cDNAs were then subcloned into pBSIISK(+) and sequenced. Primers designed to Genomescan predictions were: T2F77: 5'-GATTTCTCAAGTCACCCGCTTGGTC-3', T2F340: 5'-ACATGGGCAATGAGAACAGCACC-3', 1330F: 5'-GGAATTCCTCAGGGCCTG-3', 2330F: 5'-GGGCCCCACTCTCTCAGACAG-3', 2380R: 5'-GTCCTCCTCACCCAGCTTG-3', 3260F: 5'-AGCATGCGGTGATG-GTCAGTC-3', 3260R: 5'-CCGCATGCTCCCTCTGGC-3', 155R: 5'-GGTGGCTGTGTGCTGACCTC-3' T2R6558: 5'-CTTTGCCCTCCTGATTCTTAGCGTC-3'. As previously described in [26], rt-PCR reactions were performed from commercially available RNA from colon, prostate, and mammary gland tissue (BD Biosciences Clontech). PCR products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA) and sequenced by the Biopolymer Core Facility, Roswell Park Cancer Institute. The cDNA sequences of human long and short TACC2 isoforms have been deposited in the GenBank database with the Accession Numbers AF528099 and AF528098.

### *Immunologicals, cell lines, and Western blot analysis*

Commercial antibodies were obtained from the following companies, and used according to manufacturers' instructions: anti-TACC2 rabbit polyclonal IgG (Upstate Biotechnology, Lake Placid NY, USA), anti-BAF47 (INI-1) (BD Transduction laboratories, Lexington, KY, USA), and anti- $\beta$ -tubulin monoclonal antibody (Sigma Aldrich USA). Human embryonic kidney HEK293 cells and breast carcinoma cell lines MCF10A, T47D, HCC1395, HCC70, MCF7, MDA-MB-231, MDA-MB-435, MDA-MB-468 and SK-Br3 cells were purchased from ATCC. MCF10A

was cultured in chemically defined media [27]. The remaining cell lines were cultured in DMEM containing 10% fetal calf serum (RPCI Tissue culture media facility). Preparation of cell lysates, immunoprecipitation, and Western blot analysis were performed as previously reported [10].

### *In vitro analysis of TACC2 on tumor phenotype*

The TACC2 short isoform was fused in frame to the green fluorescent protein (EGFP) in the EGFP2 vector (BD Biosciences Clontech). This plasmid was transfected into MCF7 and MDA-MB-468, and stable transfectants selected as previously described [1]. Expression of the fusion protein was verified by fluorescence and Western blot analysis, and three stable transfectants from each cell line were then used for further analysis. In vitro analysis of transfected cell lines was carried out as previously described by Chen and colleagues [5]. The CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) was used for cell proliferation studies, according to manufacturer's instructions. Cells were plated at  $5 \times 10^3$  cells per well in 96 well plates, and the medium replaced every two days. Proliferation assays were carried out 2, 4, 6, and 8 days after initial plating. For soft agar assays, cells were seeded at  $1 \times 10^5$  cells per well in 0.35% agar in 12 well plates. Colonies 50  $\mu$ m or greater were scored as positive. Invasion assays were performed using the Matrigel Invasion Chamber assay (Becton Dickinson, Bedford, MA, USA), according to manufacturer's instructions.  $1 \times 10^5$  cells in culture medium lacking serum were introduced to the upper side of the invasion chamber. For the purpose of this experiment, culture medium containing 10% serum was used as the chemoattractant. After 24 hours at 37°C, the membrane was removed and invading cells on the lower surface fixed and stained. Cells were visualized by microscopy and counted. For all assays, each data point was performed in triplicate and differences between cell lines analyzed using one way ANOVA followed by Dunnett's Multiple Comparison Post Test (Graphpad Prism Version 3.0, Graphpad Prism Software Inc.).

### *In vitro glutathione-S-transferase pull-down assay*

The TACC2s cDNA was cloned into the GST fusion vector pGEX5X2 (Amersham Biosciences, Piscataway, NJ, USA). GST domain and GST-TACC2 proteins were expressed in *Escherichia coli* BL21(DE3)plys 'S' with 1 mM IPTG at 37°C shaker for 2 h. Cells (100 ml) were harvested and resuspended in 10 ml of 1 $\times$ phosphate buffered saline (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, protease inhibitors: 1 mM AEBSF, 0.8  $\mu$ M Aprotinin, 50  $\mu$ M Bestatin, 15  $\mu$ M E-64, 20  $\mu$ M Leupeptin, 10  $\mu$ M Pepstatin A). The cells were lysed by sonication and the lysate cleared by centrifugation at 7500 rpm at 4°C for 20 minutes. The cleared lysate was immobilized on glutathione sepharose beads. The GAS41 cDNA was cloned into



pcDNA3 (Invitrogen, Carlsbad, CA, USA) and protein synthesized by TNT quick coupled transcription/translation system kit (Promega) and radiolabeled with  $^{35}\text{S}$  methionine, according to manufacturer's instructions. About 5  $\mu\text{g}$  of immobilized GST protein was incubated with 20  $\mu\text{l}$  (40%) of the in vitro translated GAS41 protein in 750  $\mu\text{l}$  of binding buffer (20 mM Tris-HCl, pH8.0, 0.2% Triton X-100, 2 mM EDTA pH8.0, 300 mM NaCl, and protease inhibitors) at 4°C for 90 min. Unbound GAS41 was removed from the sepharose beads by washing twice with binding buffer. The beads were then washed twice with wash buffer (10 mM Tris-HCl, pH7.5, 1 mM EDTA, 400 mM NaCl, protease inhibitors) that contained 0.2% NP-40. Bound proteins were eluted from the beads by boiling in an elution buffer (100 mM Tris-HCl, pH8.0, 20 mM reduced glutathione). The proteins were analyzed on 8% SDS polyacrylamide gels. Coomassie blue staining verified equal loading of the GST fusion proteins. Dried gels were autoradiographed.

### Acknowledgments

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## The transforming acidic coiled coil proteins interact with nuclear histone acetyltransferases

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Dysregulation of the human transforming acidic coiled coil (TACC) genes is thought to be important in the development of multiple myeloma, breast and gastric cancer. However, even though these proteins have been implicated in the control of cell growth and differentiation, the mechanism by which they function still remains to be clarified. Using the yeast two-hybrid assay, we have now identified the histone acetyltransferase (HAT) hGCN5L2 as a TACC2-binding protein. GST pull-down analysis subsequently confirmed that all human TACC family members can bind *in vitro* to hGCN5L2. The authenticity of these interactions was validated by coimmunoprecipitation assays within the human embryonic kidney cell line HEK293, which identified the TACC2s isoform as a component consistently bound to several different members of HAT family. This raises the possibility that aberrant expression of one or more TACC proteins may affect gene regulation through their interaction with components of chromatin remodeling complexes, thus contributing to tumorigenesis.

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Recently, we described the cloning and genomic structure of the transforming acidic coiled coil 2 gene (TACC2) (Lauffart *et al.*, 2003), a member of the evolutionarily conserved TACC family of genes (Still *et al.*, 1999a). These genes encode proteins that are highly acidic and contain a conserved 200 amino-acid coiled coil domain, termed 'the TACC domain', which we previously predicted to play a critical role in the function of these proteins (Still *et al.*, 1999a). Both *in vitro* and *in vivo* studies indicate that the TACC proteins are functionally linked to the processes of cell growth and differentiation (Gergely *et al.*, 2000; Lauffart *et al.*, 2003; Sadek *et al.*, 2003). In fact, functional deletion of the Tacc3 gene in mice is embryonically lethal, with

homozygous knockout mice dying during mid to late gestation (Piekorz *et al.*, 2002). At present, however, evidence indicates a contrasting role for the TACC family in processes underlying the development of cancer. On the one hand, the expression of specific TACC1 isoforms has been implicated in the development of gastric cancer (Line *et al.*, 2002). On the other, the TACC1 protein has been shown to be significantly reduced in 50% of resected breast tumors, compared to normal levels, suggesting that TACC1 may be a breast tumor suppressor gene (Conte *et al.*, 2002, 2003). TACC2 has also been suggested to be a candidate tumor suppressor gene (Chen *et al.*, 2000; Conte *et al.*, 2003; Lauffart *et al.*, 2003). Overexpression of the short isoform, TACC2s, can reduce the *in vitro* tumorigenic properties of estrogen receptor negative breast cancer cell lines (Lauffart *et al.*, 2003); an effect that is apparently mediated by the conserved TACC domain (Chen *et al.*, 2000; Still *et al.*, unpublished).

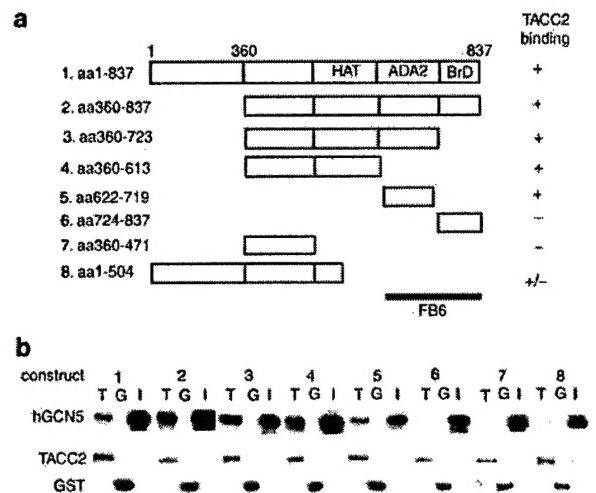
It is known that all the TACC proteins identified to date interact via the TACC domain with the microtubule-binding proteins of the stu2/msps/ch-tog family, and the Aurora Kinases, and that these interactions are required for the accumulation of ce-TAC-1, D-TACC and the vertebrate TACC3 proteins to the centrosome (Lee *et al.*, 2001; Giet *et al.*, 2002; Le Bot *et al.*, 2003; Srayko *et al.*, 2003; Tien *et al.*, 2003). However, although initially described as centrosomal proteins (Gergely *et al.*, 2000; Gergely, 2002), during interphase the human TACC proteins are actually distributed throughout the cell, with TACC1 and TACC3 being primarily concentrated in the nucleus (Gergely *et al.*, 2000). Nuclear accumulation of TACC3 has also been noted during normal mouse development (Aitola *et al.*, 2003; Sadek *et al.*, 2003). Thus, the distribution and behavior of the TACC proteins resembles those of signaling intermediates that shuttle between the cytoplasm and the nucleus, suggesting that TACC proteins may have additional functions outside their potential role at the centrosome and during mitosis. Indeed, TACC2 has been shown to migrate to the nucleus upon stimulation of microvascular endothelial cells with erythropoietin, implicating TACC2 in erythropoietin signaling (Pu *et al.*, 2001). Furthermore, we have recently demonstrated that human TACC1 and TACC2 interact with the potential nuclear oncogene, glioma

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amplified sequence 41 (GAS41) (Lauffart *et al.*, 2002, 2003) and that TACC2s is found in a complex with the INI-1 core component of the SWI/SNF complex, presumably through its interaction with GAS41 (Lauffart *et al.*, 2003). Mouse Tacc3 can coactivate the transcriptional response to hypoxia, by binding to the arylhydrocarbon nuclear translocator (ARNT1) transcription factor (Sadek *et al.*, 2000), directly implicating a TACC family member in transcriptional control. We now present further evidence for an intimate role for the TACC proteins in transcription by describing their physical association with the histone acetyltransferase (HAT) family of transcriptional coactivators. This raises the possibility that aberrant expression of one or more TACC proteins may affect gene regulation through their interaction with components of chromatin remodeling complexes, thus contributing to tumorigenesis.

To further clarify the potential function of the TACC proteins in the interphase cell, we have initiated a yeast two-hybrid based screen to identify proteins that interact with the conserved TACC domain of TACC2s. We fused the TACC2s open reading frame from amino acid (aa) 767–1094 to the GAL4 DNA-binding domain of the pAS2.1 vector. This construct was transformed into CG1945, and lack of autoactivation and lack of nonspecific interactions confirmed between the bait TACC2 protein and the GAL4 activation domain and human lamininC negative control proteins. The TACC2 bait was then used to screen a fetal brain cDNA library (BD Biosciences Clontech), as previously described (Lauffart *et al.*, 2002). Approximately  $10^6$  transformants were plated and selected on His<sup>-</sup> selective media containing 10 mM 3-aminotriazole. Of the 30 His<sup>+</sup> clones initially isolated, six proved positive when assayed for  $\beta$ -galactosidase activity using the colony lift assay. The numbers of clones isolated during this procedure were comparable to other published manuscripts, including those on TACC proteins (Conte *et al.*, 2002; Lauffart *et al.*, 2002), suggesting that these clones represented *bona fide* TACC2 interacting partners. Subsequent isolation and sequence analysis revealed that one of these clones, FB6, corresponded to the carboxy terminal 215 aa's of the HAT hGCN5L2 (Figure 1a). This region contains the bromodomain (BrD), which has been identified in several transcriptional regulatory proteins, and the ADA2 interaction region (Xu *et al.*, 1998) (Figure 1a). To confirm that the interaction between hGCN5L2 and TACC2s is direct, and to further refine the binding site of TACC2s on hGCN5L2, we next assessed this novel interaction using the glutathione-S-transferase (GST) pull-down assay. Deletion and smaller fragments of hGCN5L2 were cloned in pET28c and tested for their ability to interact with TACC2s. TACC2s was expressed in *E. coli* as a GST fusion protein, incubated with each *in vitro* translated hGCN5L2 subfragment, and subjected to several wash steps to remove any nonspecific binding. As can be seen in Figure 1b, TACC2s was found to bind specifically to the ADA2 interaction region, but was unable to interact with the BrD, when this region was expressed separately. Thus, the interaction of TACC2



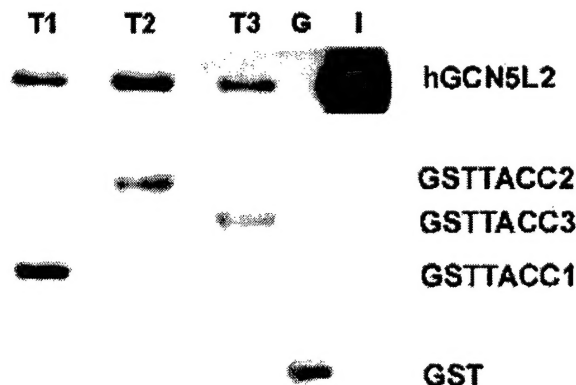
**Figure 1** *In vitro* interactions between TACC2s and hGCN5L2. (a) Diagram and summary of deletion constructs for hGCN5L2-binding domain mapping. Extents of the constructs are defined by the reference protein sequence NP\_066564. The original clone identified by yeast two-hybrid (FB6) included the ADA2 interaction domain and the BrD. (b) Interaction of TACC2 with hGCN5L2 constructs by GST pull-down. Lane numbering corresponds to the numbered hGCN5L2 construct in (a). Top panel: Autoradiograph of 12% SDS polyacrylamide gels with *in vitro* translated hGCN5L2 construct pulled down with GST-TACC2s (T) or GST (G). I: 5% input of hGCN5L2 construct; bottom two panels represent Coomassie blue stained gels of the pull-down experiment, verifying equal loading of the GST fusion proteins. Full-length TACC2s was cloned into pGEX5X2 (Amersham Biosciences, Piscataway, NJ, USA), and GST-fusion protein prepared and immobilized on glutathione sepharose beads, as previously described (Lauffart *et al.*, 2003). Subclones of hGCN5L2 were generated by PCR (primers sequences available on request) and cloned into pET28c (Novagen, Madison WI, USA). These constructs were used to synthesize <sup>35</sup>S-radiolabeled proteins by the TNT quick coupled transcription/translation system kit (Promega, Madison WI, USA), according to the manufacturer's instructions. In total, 5  $\mu$ g of immobilized GST protein was incubated with 60% of the *in vitro* translated protein in HEPES binding buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 12% glycerol and protease inhibitors) at 4°C for 90 min. Unbound labeled protein was removed by washing four times with binding buffer. Bound proteins were then eluted by boiling in GST elution buffer (100 mM Tris-HCl, pH 8.0, 20 mM reduced glutathione) and analysed on 12% SDS polyacrylamide gels. Dried gels were autoradiographed.

with the yeast two-hybrid clone FB6 was due to the specific binding of the coiled coil domain of TACC2 to the ADA2 interacting domain contained within this clone. In addition, a second TACC2-binding domain in hGCN5L2 was also detected: TACC2s interacted with those constructs that contained the intact HAT domain, and bound weakly to construct 8, which contained the N-terminal section of the HAT domain. Lack of binding to aa 360–471 further suggested that TACC2 specifically binds to the ADA2 interaction domain and the intact HAT catalytic domain of hGCN5L2.

The TACC family of proteins is defined by a highly conserved C-terminal 200 aa coiled coil domain (TACC domain) (Still *et al.*, 1999a, b). The TACC domain of TACC2 shows highest homology to that of TACC1

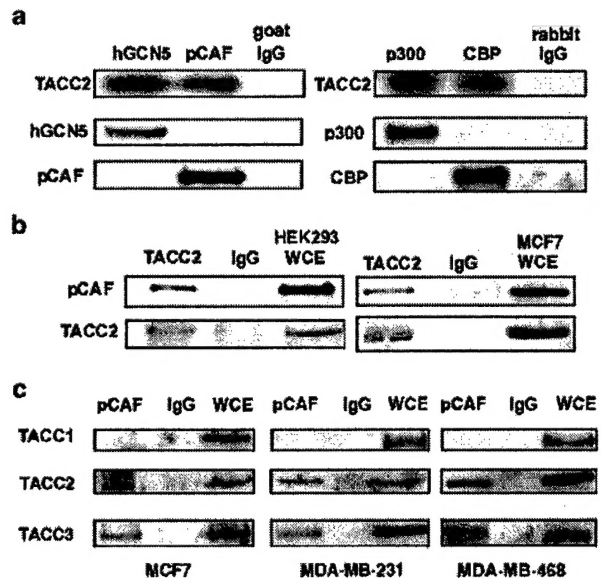
(74% identity) (Still *et al.*, 1999a). Thus, the finding that this region of TACC2 binds to hGCN5L2 suggested that the other TACC family members could also bind to this HAT. To address this issue, TACC1 (aa596-ter) and TACC3 (aa116-ter) were fused to GST and assayed for binding to *in vitro* synthesized hGCN5L2, as described above. Figure 2 shows that both these proteins bind directly to the hGCN5L2 protein, confirming that the TACC proteins, as a whole, are a family of hGCN5L2-binding proteins.

Given that the TACC2s-binding site on hGCN5L2 is highly related to pCAF (79% identity), it would seem likely that TACC2s could also interact with pCAF. Furthermore, previously, Yang *et al.* (1996) demonstrated that pCAF and hGCN5L2 form complexes with two other highly related HATs, that is, the CREB-binding protein (CBP) and p300, and different combinatorial interactions between these proteins can govern the transcriptional activation of a number of promoters by specific transactivators. Thus, to further examine whether native TACC2s can be found complexed with other HATs *in vivo*, we next performed coimmunoprecipitation experiments with commercial antibodies individually raised against native hGCN5L2, pCAF, p300 and CBP. Figure 3a demonstrates that in the human embryonic kidney cell line 293, native TACC2s coimmunoprecipitates with all these four proteins. We next examined whether a highly specific commercial antibody raised to an internal region of human TACC2s (Lauffart *et al.*, 2003), which does not overlap the proposed HAT-binding site, could immunoprecipitate the native TACC2-pCAF complex from HEK293 and the breast cancer cell line MCF7. In both cell lines, this antibody immunoprecipitates a TACC2 complex that contained endogenous pCAF (Figure 3b), further strengthening the conclusion that TACC2s binds to pCAF in human cells.



**Figure 2** *In vitro* interaction between hGCN5L2 and the TACC proteins. Top panel: Autoradiograph of 10% SDS polyacrylamide gel with *in vitro* translated hGCN5L2 construct pulled down with GST-TACC1 aa596-ter (T1), GST-TACC2s (T2) aa2-ter, GST-TACC3 aa116-ter (T3) or GST (G). I: 5% input of *in vitro* translated hGCN5L2 construct. The bottom two panels represent Coomassie blue stained gels of a pull-down experiment showing loading of GST-TACC proteins and GST. Assays were performed as described in Figure 1

We have previously demonstrated that overexpression of TACC2s can reduce the *in vitro* tumorigenic properties of some breast cancer cell lines (Lauffart *et al.*, 2003), a process that appears to be mediated by the conserved TACC domain (Chen *et al.*, 2000; Still *et al.*, unpublished). Furthermore, the involvement of HATs in human cancer has now been demonstrated (Borrow *et al.*, 1996; Ida *et al.*, 1997; Sobulo *et al.*, 1997; Chaffanet *et al.*, 1999; Gayther *et al.*, 2000), and the hGCN5L2 gene has been suggested to be a candidate tumor suppressor in those cases of ovarian and breast cancer that show loss of heterozygosity in chromosome 17q21, without BRCA1 mutations (Tangir *et al.*, 1996; Niederacher *et al.*, 1997). Therefore, we were particularly interested in determining whether the TACC proteins could interact with hGCN5L2 and pCAF

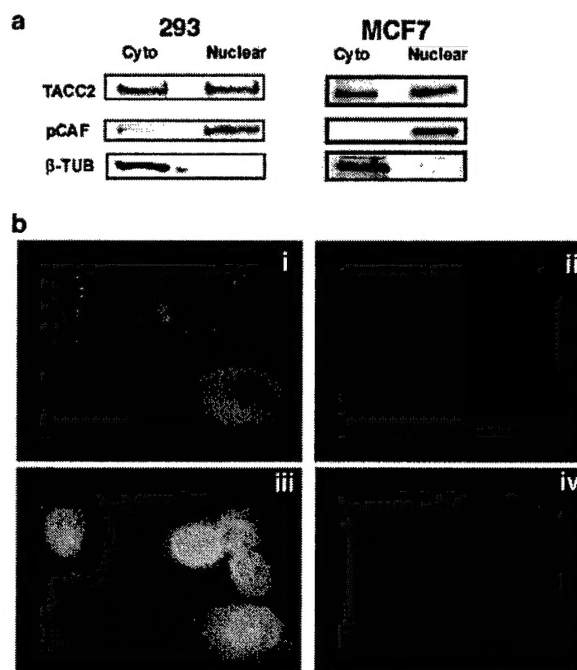


**Figure 3** TACC proteins interactions with HATs in cells. (a) HEK293 lysates were immunoprecipitated with goat antibodies to native hGCN5L2, and pCAF, or rabbit polyclonal antibodies to CBP, and p300, or normal species specific IgG, and filters incubated with  $\alpha$ -TACC2 antibody. TACC2s is detected in the immunoprecipitates (IPs) of the four HATs, but not in control IgG IPs. (b) pCAF is specifically immunoprecipitated from HEK293 and MCF7 cell lysates by a commercial antibody raised to TACC2. (c) pCAF interacts with TACC2s and TACC3 in breast cancer cells. Cells were immunoprecipitated with mouse  $\alpha$ -pCAF or mouse IgG and immunoblotted with  $\alpha$ -TACC1,  $\alpha$ -TACC2 or  $\alpha$ -TACC3 antibody as described in Lauffart *et al.* (2002). The  $\alpha$ -pCAF antibody specifically immunoprecipitates both the native TACC2s and TACC3 protein in MCF7, MDA-MB-468 and MBA-MB-231, but fails to immunoprecipitate TACC1. WCE: whole-cell extract. Commercial antibodies were obtained from the following companies, and used according to the manufacturers' instructions: anti-TACC2 rabbit polyclonal IgG #07-228 (Upstate Biotechnology, Lake Placid NY, USA), anti-hGCN5 (#sc-6303), anti-pCAF (#sc-6300, sc-13124), anti-p300 (#sc-584) and anti-CBP (#sc-369) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Normal IgG and anti-goat-HRP, anti-rabbit-HRP and anti-mouse-HRP conjugates were also obtained from Santa Cruz Biotechnology. The culture of cell lines, preparation of cell lysates, immunoprecipitation and Western blot analysis were performed as described in Lauffart *et al.* (2002)

expressed in breast cancer cell lines. All of the MDA-MB-231, MDA-MB-468 and MCF7 cell lines expressing TACC2s (Lauffart *et al.*, 2003) also express pCAF (Figure 3c). However, since these cell lines do not express significant levels of hGCN5L2 (data not shown), our coimmunoprecipitation analysis was carried out exclusively using an antibody specific for native pCAF. Consistent with our prediction, both native TACC2s and TACC3 were found in the complex containing endogenous pCAF (Figure 3c). However, we were unable to detect any interaction between TACC1 and pCAF in any of these cell lines (Figure 3c), or the immortalized, but not transformed MCF10A breast epithelial cell line (data not shown). Thus, it remains uncertain whether in breast epithelial cells, TACC1 is a preferred binding partner of this HAT.

As stated above, and shown in Figure 4, TACC2 is distributed throughout the cytoplasm and the nucleus of cells in culture. To further confirm that the pCAF and TACC2 proteins could physically interact in the intact cell, we next determined whether pCAF colocalized with endogenous TACC2 in the breast cancer cell line MCF7. Indirect immunofluorescence microscopy demonstrated that native TACC2 is found in both cytoplasmic and nuclear compartments of interphase MCF7 cells (Figure 4b). This distribution has been previously observed for TACC2 in other human cell lines (Chen *et al.*, 2000; Gergely *et al.*, 2000). In these cells, endogenous pCAF is diffusely distributed throughout the nucleus, but is absent from the nucleoli (Figure 4b). Superimposing the images for the two antibodies reveals that both proteins colocalize throughout the nucleus, but are not found in discrete subnuclear structures. This indicates that both the TACC proteins and the HATs can be physically located and potentially interact in the same subcellular compartment.

As presented above, we have demonstrated that the TACC proteins have the ability to bind *in vitro* and in cellular systems to the members of the HAT family of transcriptional coactivators. In humans, hGCN5L2 copurifies with the SAGA-related transcriptional adaptor complexes STAGA and TFTC (Martinez *et al.*, 1998). The homologous protein, pCAF, also coimmunoprecipitates with factors known to regulate the transcriptional activity of promoters (Ogryzko *et al.*, 1998). In these complexes, the HAT activity of hGCN5L2/pCAF is required for the acetylation of the N-terminal tails of core histones and/or transcription factors, which facilitates the binding of the general transcription machinery, thereby promoting transcription. Thus, the direct interactions of members of the TACC family with the transcription factors ARNT1 (Sadek *et al.*, 2000) and STAT5 (Piekorz *et al.*, 2002), the oncogene GAS41 (Lauffart *et al.*, 2002, 2003), the SWI/SNF core component INI1 (Lauffart *et al.*, 2003), and now the HAT proteins, further support the notion that the TACC family may represent a novel family of transcriptional accessory or regulatory proteins. Indeed, *in vivo*, immunohistochemical analysis of cross sections of normal human and mouse tissues clearly show that the major subcellular site of one TACC protein,



**Figure 4** Colocalization of TACC2s and pCAF endogenous proteins in the nucleus of interphase cells. (a) TACC2 is distributed in both nuclear and cytoplasmic (cyto) extracts of HEK293 cells and MCF7 cells. pCAF is predominantly found in the nucleus. The leakage of pCAF protein into the cytoplasmic fraction of HEK293 cells is probably due to nuclear breakdown during extract preparation. Cytoplasmic and nuclear extracts were prepared as described in Schreiber *et al.* (1989). (b) (i) Indirect immunofluorescence of TACC2 detected with fluorescein isothiocyanate (FITC)-conjugated secondary antibody shows distribution in the cytoplasm and nucleus of MCF7 cells; (ii) nuclear localization of pCAF, detected with Texas red-secondary antibody; and (iii) overlay of the previous images reveals colocalization of both proteins in the nucleus. The green and red colors in the superimposed images result in a yellow color. Nuclei were counterstained with DAPI (panel iv). MCF7 cells were prepared as previously described (Lauffart *et al.*, 2002). Cells on coverslips were incubated with the  $\alpha$ -TACC2 polyclonal antibody (Upstate Biotech) together with the  $\alpha$ -pCAF mouse monoclonal antibody (Santa Cruz) for 1 h at room temperature. The coverslips were washed with PBS (3  $\times$  5 min.) and incubated with a mixture of FITC-anti-rabbit IgG and Texas red-conjugated anti-mouse IgG for 30 min. Finally, the coverslips were washed with PBS (3  $\times$  5 min), mounted with Vectashield containing DAPI and examined at  $\times$  60 magnification (oil). Secondary antibodies were obtained from Jackson Immunolabs

TACC3, is the nucleus indicating that this may be the site of the major function of this protein in interphase cells (Aitola *et al.*, 2003; Sadek *et al.*, 2003).

It is also clear that the function of hGCN5L2 and pCAF is not limited to transcription. hGCN5L2/pCAF also interact with pre-mRNA splicing complexes and DNA-damage repair enzymes, suggesting that acetylation by these HATs is of critical importance to a diverse set of nuclear regulatory events (Martinez *et al.*, 2001). The lack of significant binding of pCAF to TACC1 in breast cancer cell lines may in part be due to a more prominent role of TACC1 in post-transcriptional events, through its interaction with the RNA processing and



transport proteins, LSm7 and SmG (Conte *et al.*, 2002; Lauffart *et al.*, 2002), as opposed to transcriptional regulation. Intriguingly, a post-transcriptional role for the *Xenopus* TACC protein, maskin, has previously been demonstrated (Stebbins-Boaz *et al.*, 1999). This protein is involved in the regulation of the specific polyadenylation-induced translation of maternal mRNA during oocyte maturation (Stebbins-Boaz *et al.*, 1999), suggesting that the vertebrate TACC proteins may be directly involved at multiple steps during the process of gene expression.

In summary, the findings presented here, together with those from previous studies, clearly indicate that TACC proteins are a multifunctional family of proteins that not only play a role in the functional aspects of mitotic spindle formation during mitosis but may also perform an assembly or coordination function bringing elements of the chromatin remodeling, transcriptional and post-transcriptional machinery together in the interphase nucleus. Based on the results presented in this manuscript and the growing number of basal transcription components with which the TACC

proteins interact, it is tempting to speculate that the interaction of TACC proteins with the SWI/SNF complex and HATs may in part explain the coactivation of hypoxia-induced transcription that has been observed for murine TACC3 (Sadek *et al.*, 2000). Furthermore, this may suggest that the ability of TACC2s to reduce the malignant properties of some breast carcinomas may in part be due to changes in the activity of TACC2-HAT complexes in the regulation of genes governing cell proliferation and metastasis.

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